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Role of Lipids in the Biosynthesis of the Bacterial Cell Envelope

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INTRODUCTION

In the early 1950's, Salton elevated the bacterial cell envelope from a vague geographic expression to a respectable cell organelle by developing techniques of isolation, thereby making the structure available for classic biochemical study. This led to an outburst of activity during the next decade until, by the mid-1960's, the major specific macromolecular components of the cell envelope—peptidoglycan, teichoic acid and lipopolysaccharide (LPS)—were known and their chemical structures were fairly well understood. All three macromolecules were known to be complex polysaccharides, and although many structural details remained to be worked out, sufficient information was available to prompt several laboratories to begin studies of their biosynthesis (68).

Between 1955 and 1957, Leloir and his collaborators made the important discovery that uridine diphosphate (UDP)-glucose was the glucosyl donor in the biosynthesis of several oligosaccharides (11, 28) and of glycogen (29), and this soon led to the demonstration that nucleotide sugars acted as glycosyl donors in several other polysaccharide-synthesizing systems. Therefore, it was generally assumed that nucleotide sugars would also be involved in the biosynthesis of complex polysaccharides such as the polymers of the bacterial cell envelope. This expectation was confirmed by the demonstration that nucleotide sugars were intermediates in the biosynthesis of pneumococcal capsular polysaccharides (74).

Studies in several laboratories then showed that enzyme activities in the cell envelopes of *Escherichia coli* and *Salmonella typhimurium* utilized UDP-galactose, UDP-glucose and UDP-*N*-acetyl glucosamine as glycosyl donors in synthesis of the lipopolysaccharides that are components of the cell envelopes of all gram-negative bacteria (15, 40, 46, 47), and shortly thereafter nucleotide sugars were also shown to be involved

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in biosynthesis of peptidoglycans and teichoic acids. In studies of peptidoglycan biosynthesis, Chatterjee and Park (12) and Meadow et al. (37) showed that cell envelope preparations of *Staphylococcus aureus* contain enzymes that transfer glycosyl residues from nucleotide sugars into the polysaccharide backbone of the peptidoglycan molecule. Similarly, teichoic acid biosynthesis was shown to involve cytidine diphosphate (CDP)-ribitol and CDP-glycerol as precursors of the ribitol-P and glycerol-P moieties of the polysaccharide backbone (7). Finally, in studies of glycosyldiglycerides from *Micrococcus lysodeikticus* and *Diplococcus pneumoniae* (23, 77) and of a rhamnosyl glycolipid from *Pseudomonas aeruginosa* (8, 9), it was demonstrated that nucleotide sugars were the active glycosyl donors in the biosynthesis of several glycolipid components of the cell envelope.

The general role of nucleotide sugars was firmly established, therefore, and except for the annoying complication that all of the enzyme activities were particulate and thus not amenable to conventional purification procedures, the major outlines of biosynthesis of the components of the cell envelope seemed clear.

The fact that the enzyme activities were particulate, however, proved to be of critical importance. A large number, perhaps the majority, of cellular enzymes are "particulate." In nearly all cases, particulate means membrane-bound, and the realization that the enzymes of cell envelope biosynthesis were membrane enzymes provided the clue to the next major advance in this field, namely the discovery of the essential role of membrane lipids in the biosynthetic reactions.

Two mechanisms of participation of membrane lipids are now known, involving two entirely different types of lipid. In the first, the active lipids are glycerophosphatides such as phosphatidylethanolamine (PE), and the lipids act as physical cofactors, activating the reactions but not themselves participating in the biosynthetic reaction sequence. This mechanism has been shown to operate in synthesis of the core region of lipopolysaccharide and is also of importance in many other membrane enzyme reactions in bacterial and animal cells. The second mechanism utilizes a unique class of membrane lipids, the polyisoprenoid alcohol phosphates. These compounds are present in trace amounts, comprising less than 0.5% of the membrane lipids in *Salmonella typhimurium*, but they play a key role in cell wall biosynthesis. The lipids are called carrier lipids and they participate directly in the biosynthetic sequence by formation of covalently linked lipid-glycosyl intermediates. The carrier lipid mechanism has now been shown to play a role in the biosynthesis of several cell envelope components including peptidoglycan (1), the O-antigen region of LPS (79, 84), a mannan of *M. lysodeikticus* (70), a capsular polysaccharide from *Aerobacter aerogenes* (17), and the teichoic acid of *Staphylococcus lactis* (14). Several observations also suggest that a similar mechanism may be involved in the biosynthesis of mannan in yeast (78), of a mannose-containing cell wall polysaccharide of mung beans (24), of glycoproteins in rabbit liver (10), and of glucose-containing polymers in pig liver (3).

ABBREVIATIONS

Among the abbreviations used in this article are the following: Gal, D-galactosyl; Glc, D-glucosyl; GlcNAc, *N*-acetyl-D-glucosaminyl; GlcUA, D-glucuronosyl; Abe, abequosyl (3,6-dideoxy-D-galactosyl); MurNAc-pentapeptide, *N*-acetyl-D-muramyl-L-alanyl-γ-D-glutamyl-L-lysyl-D-alanyl-D-alanine.

LPS STRUCTURE

The LPS of gram-negative bacteria are complicated molecules, containing a lipid portion (Lipid A) covalently linked to a complex polysaccharide. The polysaccharide can, in turn, be subdivided into two regions, the core and the O-antigen. The core consists of a nonrepeating series of sugar residues and appears to be generally similar in most gram-negative bacteria. In contrast, the O-antigen is composed of a repeating array of identical oligosaccharide units. The detailed structure of the O-antigen varies from strain to strain, and this variation forms the basis for the serological classification of the enterobacteria (32). The difference in structure between core and O-antigen regions is reflected in a corresponding difference in the biosynthetic pathways, with glycerophosphatides required for core biosynthesis whereas glycosyl carrier lipids participate in synthesis of the O-antigen.

Each LPS molecule is thought to consist of several of the basic units indicated in Fig. 1, held together by covalent cross-links (45). Until recently, the true molecular weight and hence the number of units per molecule had not been determined because of the marked tendency of the LPS to aggregate in both polar and nonpolar solvents. However, evidence has now emerged that in two species of *Salmonella* the average LPS molecule contains approximately three polysaccharide chains (n = 3 in Fig. 1). This was first shown by the studies of Malchow et al. (35) on an enzymatically deacylated LPS from *Salmonella london*. Sedimentation velocity studies of the deacylated preparation indicated an average of 2.6 polysaccharide chains per molecule. More recently Romeo et al. (58) were able to determine

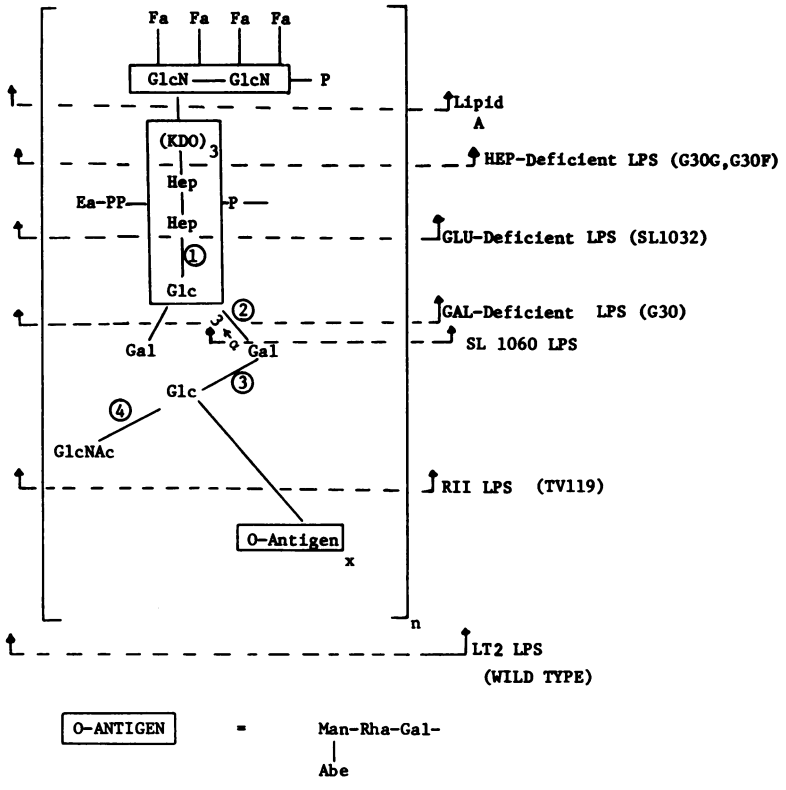


FIG. 1. *LPS of Salmonella typhimurium [adapted from Osborn (45)]. The points of termination of the incomplete LPS of various rough mutants are indicated. The numbers indicate the sites of action of four of the glycosyl transferase enzymes involved in biosynthesis of the core. (1) UDP-glucose:LPS glucosyl transferase I (64); (2) UDP-galactose:LPS α,3-galactosyl transferase (64); (3) UDP-glucose:LPS glucosyl transferase II (46); (4) UDP-N-acetylglucosamine:LPS N-acetylglucosaminyl transferase (46).*

the molecular weight of the undegraded LPS of *S. typhimurium* G-30 (*see* Fig. 1). The problem of molecular aggregation was overcome by first acetylating the molecule, thereby making it highly soluble in benzene. The molecular weight was then determined by the technique of sedimentation equilibrium, with benzene as the solvent, and a molecular weight of 10,300 ± 650 was calculated for the nonacetylated LPS. Since the expected molecular weight of a single polysaccharide chain and lipid A moiety is approximately 3,350, each LPS molecule appears to contain three of the basic units shown in Fig. 1.

As shown in Fig. 1, the LPS molecule contains a highly polar polysaccharide region and a large nonpolar lipid portion (Lipid A). Therefore, LPS are amphipathic molecules and have a strong tendency to associate with other amphipathic molecules, such as phospholipids, to form mixed micelles and other liquid crystalline structures. This amphipathic character plays a major role in promoting phospholipid-LPS interactions within the membrane and is a key to understanding the involvement of membrane phospholipids in LPS biosynthesis.

It has been known for many years that the lipopolysaccharides of gram-negative bacteria are located in their cell envelope fraction. The gram-negative cell envelope contain two distinct membranes, an inner (cytoplasmic or plasma membrane) and an outer membrane, each having a characteristic "unit membrane" structure revealed by electron microscopy. A substantial body of evidence now indicates that the lipopolysaccharides are membrane components, mainly located in the outer membrane of the cell envelope together with phospholipids and proteins (38, 50, 66, 71).

BIOSYNTHESIS OF THE CORE REGION OF BACTERIAL LPS

A group of membrane-bound enzymes are involved in biosynthesis of the core region of bacterial LPS. Each enzyme catalyzes transfer of a single sugar residue from a nucleotide sugar precursor to the nonreducing end of the growing

polysaccharide chain. This sequential transfer of monosaccharide units results in growth of the polysaccharide, eventually resulting in the "mature" rough (RII) LPS (*see* Fig. 1).

The unravelling of the pathways of biosynthesis of the core region was made possible by the availability of mutant bacteria blocked in synthesis of specific nucleotide sugars. Nikaido and Fukasawa (42) first demonstrated that organisms with deficiencies in nucleotide sugar biosynthesis produce incomplete LPS by showing that mutants of *S. typhimurium* deficient in synthesis of UDP-galactose contained LPS that lacked galactose and several other sugars normally present in the LPS of the wild type (Fig. 1; G-30 LPS). Soon thereafter, mutants deficient in synthesis of UDP-glucose and guanosine diphosphate (GDP)-mannose were studied and were also found to produce incomplete LPS (16*a*, 16*b*, 17*a*, 60, 76*a*). In subsequent studies, the presence of the core transferase enzymes was demonstrated directly by using the incomplete mutant lipopolysaccharides as acceptors for sugar transfer in the enzyme reactions. These studies demonstrated that the transferases and the lipopolysaccharides which act as acceptors in the reactions are both present in the cell envelope fraction. By the sequential addition of the proper nucleotide sugars to the cell envelope preparations, it was shown that a series of enzymes catalyze the sequential incorporation of glucose, galactose, glucose, and *N*-acetylglucosamine into the growing polysaccharide chain (15, 46). All of these enzymes are recovered in the cell envelope fraction and fall in the general group of membrane-bound enzymes.

Since the cell envelope also contains large amounts of LPS, phospholipids, proteins, ribonucleic acid (RNA), and peptidoglycan, detailed studies of the reactions in the intact membrane are difficult because of the large number of components present in the particulate enzyme preparations. In the case of two of the transferase enzymes, however, this difficulty was overcome when it was shown that a portion of the enzyme activities was present in the soluble fraction after sonic treatment of the cells (64). The two enzymes catalyze the transfer of glucose and galactose from UDP-glucose and UDP-galactose, respectively, into the deficient LPS of appropriate mutant strains.

$$\text{UDP-Glc} + \text{glucose-deficient LPS} \rightarrow \text{Glc-LPS} + \text{UDP} \quad \text{I}$$

$$\text{UDP-Gal} + \text{galactose-deficient LPS} \rightarrow \text{Gal-LPS} + \text{UDP} \quad \text{II}$$

In reaction I (UDP-glucose:LPS glucosyl transferase), the glucose-deficient acceptor LPS is obtained from a mutant unable to synthesize UDP-glucose. For reaction II (UDP-galactose: LPS-α,3 galactosyl transferase), the galactose-deficient acceptor LPS comes from a mutant defective in the synthesis of UDP-galactose. The soluble enzyme activities were detected by measuring the ability of the enzyme preparations to catalyze the transfer of ^{14}C-galactose or ^{14}C-glucose from the appropriate nucleotide sugar into the deficient LPS of a crude cell envelope preparation. In these experiments the cell envelope was first heated to inactivate any endogenous transferase enzyme activity. When assayed in this way, the soluble fraction obtained after sonic disruption of the cells contained significant amounts of both glucosyl and galactosyl transferase activities. In both cases, the sugar was transferred into covalent linkage to the acceptor LPS of the cell envelope. However, a paradox soon became apparent; although the LPS in the cell envelope was an active acceptor, the purified LPS themselves, when separated from the other components of the cell envelope, did not act as acceptors in the transferase reactions. This suggested that a requirement might exist for an additional component of the cell envelope.

ROLE OF GLYCEROPHOSPHATIDES IN LPS BIOSYNTHESIS

Requirement for Membrane Lipids

The first hint that membrane lipids might be involved in LPS biosynthesis came from the observation that extraction of the cell envelope with lipid solvents completely destroyed its ability to act as acceptor for sugar transfer (62). Nearly all of the cell envelope lipids are removed by the extraction procedure, leaving the LPS

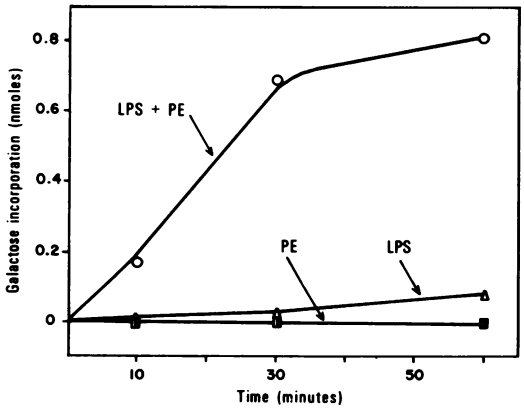


FIG. 2. *Effect of lipid on the galactosyl transferase reaction. Incubation mixtures contained UDP-^{14}C galactose, soluble enzyme, and acceptor as indicated. LPS, galactose-deficient lipolysaccharide; PE, phosphatidylethanolamine.*

behind in the residue together with the other nonlipid components of the intact cell envelope. When the extracted lipid was recombined with the cell envelope residue, the acceptor activity was completely restored. This suggested that the lipid was recombining with residual LPS or other cell envelope components, or with both, to restore the original structure. The specific role of the lipids became clearer when it was shown that the cell envelope lipids were also able to convert purified LPS into effective acceptors in the glucosyl transferase and galactosyl transferase reactions, although the isolated LPS did not act as glycosyl acceptors in the absence of added lipid (62; Fig. 2). This ruled out the participation of still other cell envelope components and made it possible to study the reactions in a completely defined system, using purified transferase enzymes, lipids, and LPS (16).

Structural Requirements of the Lipid Molecule

The component responsible for the activity of the cell envelope lipids in the transferase reactions proved to be PE. This was shown by fractionating the total lipid extract and demonstrating that over 90% of the activity of the crude extract was recovered in the purified PE fraction. Studies of various synthetic and natural compounds revealed that other phospholipids also were active in the transferase reactions and that certain structural characteristics of the lipid molecule are required for activity (65). The active lipids are glycerophosphatides and the entire α-glycerophosphate backbone of the molecule is necessary for activity. Thus, dioleylglycerol is inactive, whereas dioleylphosphatidic acid is highly active. Many other nonglycerophosphatides have been tested and are inactive.

Within the general class of glycerophosphatides, specificity resides both in the nonpolar and in the polar portions of the molecule. Full activity requires that the molecules contain unsaturated or cyclopropane fatty acyl groups (R^1 and R^2 in Fig. 3), and conversion of the acyl residues from unsaturated to saturated by catalytic hydrogenation causes complete loss of activity. Two residues are required as shown by the lack of activity of lysophosphatides (monoacylphosphatides) prepared from fully active diacylphosphatides. The significance of the requirement for unsaturated or cyclopropane groups will be discussed later, but it should be noted here that the activity of phospholipids containing cyclopropane fatty acids eliminates the possibility that the unsaturated acyl residues participate directly in the transferase reactions by virtue of the chemical reactivity or electronic configuration of their double bonds.

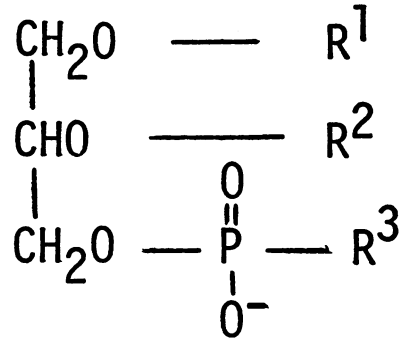


FIG. 3. *Structure of glycerophosphatides.*

The polar portion of the phosphatide molecule (R^3 in Fig. 3) also imparts specificity in the transferase reactions (65). For example, although various groups can replace phosphorylethanolamine in the R^3 position, phosphatidylcholines are uniformly inactive in the galactosyl transferase reaction. Choline differs from ethanolamine by the presence of three *N*-methyl groups in place of the free amino group of the ethanolamine residue. As might be expected, partially methylated phosphatidylethanolamines (phosphatidyl-*N*-methylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine) show intermediate activities between PE and phosphatidylcholine. These differences in activity probably reflect steric factors related to the size and state of hydration of the bulky methyl groups. Changes in charge of the head groups of the phospholipids do not appear to be responsible for the effects (65).

LPS-Phospholipid-Enzyme Interactions

The major role of the phospholipids is to physically interact with LPS, thereby converting the LPS into an active participant in the transferase reactions. The active substrate is therefore a LPS-phospholipid complex in which the components are held together by noncovalent bonds.

The first evidence for such an interaction was the observation that the galactosyl transferase reaction requires that LPS and PE be heated together prior to addition of enzyme and nucleotide sugar. When the components were heated separately no activity was seen, suggesting that the heating promoted an interaction between the two components leading to formation of a LPS-phospholipid complex which was the true acceptor in the reaction. This interpretation was confirmed by the subsequent isolation of the postulated LPS-PE complex.

There is a marked difference in the buoyant densities of PE and LPS, and this makes it possible to isolate complexes of the two components by

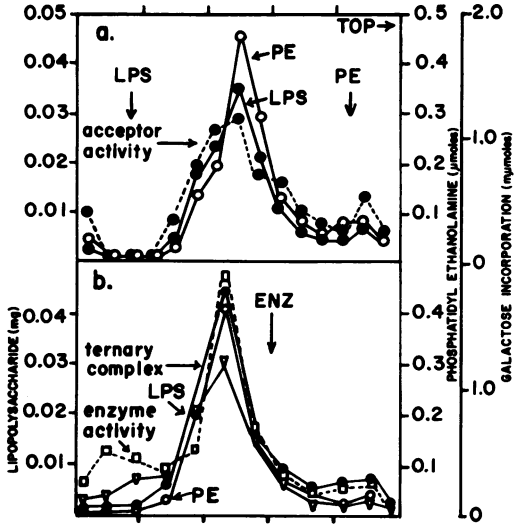


FIG. 4. *Isolation of (a) LPS·PE and (b) enzyme. LPS·PE complexes by isopycnic centrifugation [from Weiser and Rothfield (80)]. Mixtures of LPS and PE were heated to 60 C for 30 min and were slowly cooled to room temperature before being applied to the gradient. Acceptor activity as assayed by adding UDP-^{14}C galactose and galactosyl transferase enzyme to each tube; enzyme activity was assayed by adding excess LPS-PE acceptor and UDP-^{14}C galactose to each tube. Ternary complex was assayed by adding UDP-^{14}C galactose to each tube. Arrows indicate the positions of LPS, PE, or ENZ when centrifuged under the same conditions but in the absence of the other two components.*

isopycnic density gradient centrifugation (80). When mixtures of galactose-deficient LPS and of PE are heated and slowly cooled and are then subjected to gradient centrifugation to equilibrium, a new peak of intermediate density appears, containing both components (Fig. 4a). When the heating and cooling procedure is omitted, most of the LPS remains uncomplexed and is recovered at the bottom of the gradient. The isolated binary complex (LPS·PE) is then capable of binding the galactosyl transferase enzyme, as shown by the isolation of a ternary complex (enzyme·LPS·PE) by the same gradient centrifugation technique (Fig. 4b). The isolated ternary complex is fully active in the transferase reaction when UDP-galactose is added. Binding of the transferase enzyme is quite specific since the bulk soluble proteins of *S. typhimurium* do not bind to the binary complex. The following sequence of reactions, therefore, describes the reconstitution of the galactosyl transferase system.

$$\text{LPS} + \text{PE} \rightarrow \text{LPS·PE} \qquad \text{III}$$

$$\text{LPS·PE} + \text{enzyme} \rightarrow \text{enzyme·LPS·PE} \qquad \text{IV}$$

$$\text{Enzyme·LPS·PE} + \text{UDP-galactose} \rightarrow \text{galactosyl-LPS·PE} + \text{UDP} (+ \text{enzyme}) \qquad \text{V}$$

Since the enzymatic properties of the reconstituted complexes are identical to those of the native cell envelope, it is likely that the organization of molecules in the in vitro complexes is similar to their organization in the intact membrane.

The binary and ternary complexes each contain large numbers of molecules. This reflects the fact that both LPS and PE are amphipathic compounds and therefore tend to form organized multimolecular structures when dispersed in aqueous media. Electron microscopic and X-ray diffraction studies of purified LPS (6, 30, 63) and PE (33, 63) suggest that, under the conditions of the experiments, these structures are primarily bimolecular leaflets. This led to the proposal that the binary LPS·PE complexes may represent *mixed* bimolecular leaflets containing large numbers of both molecules (Fig. 5). Electron microscopic studies of the binary complexes were consistent with this view, although far from conclusive (63), but further support for the hypothesis has recently emerged from studies involving monolayer techniques.

Monolayer Experiments

One approach to the study of the molecular organization of a reconstituted membrane system is to use a technique in which the molecules are arranged in a two-dimensional array resembling the planar structure of the membrane. One such method is the monolayer technique and this was

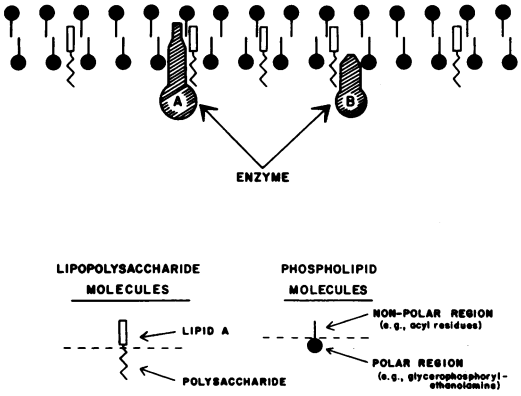


FIG. 5. *Hypothetical model of LPS, PE, and transferase enzyme in the membrane. A and B represent alternate possibilities. In B, the enzyme extends into the core region of the polysaccharide but does not penetrate into the nonpolar (hydrocarbon) region of the membrane; in A, the enzyme extends into the nonpolar region.*

recently applied successfully to the galactosyl transferase system (58, 59). In this method a monomolecular film of phospholipid is formed on the surface of an aqueous subphase. The film consists of a single layer of phospholipid molecules, arranged side-by-side with nonpolar portions facing upward and the polar head groups facing the subphase solution (Fig. 6). Penetration of molecules into the monolayer is indicated by changes in surface pressure. The film can also be removed for direct analysis, and molecular areas can be obtained by simple calculation.

This technique was used to sequentially incorporate LPS and enzyme protein into a phospholipid monolayer. A monomolecular film of PE was first formed on the surface of an aqueous subsolution and ^{3}H-labeled LPS was injected beneath the surface. Penetration of LPS into the phospholipid monolayer was indicated by an increase in surface pressure (Fig. 7) and was confirmed by direct analysis of the film.

The mixed LPS-PE monolayer was similar to the native acceptor in the cell envelope in its ability to serve as an active substrate in the galactosyl transferase reaction without preliminary heating. In this respect the binary film resembled the cell envelope of mutant strains such as *S. typhimurium* SL1060 (16, 44; Fig. 1), which contain the normal LPS-PE acceptor but which lack the galactosyl transferase enzyme. In contrast, as discussed above, simple mixtures of LPS and PE in aqueous suspension must be heated and slowly cooled together to form a functional acceptor. These results strongly support the idea that the arrangement of LPS and PE molecules in the mixed monolayer is similar to their arrangement in the intact membrane structure.

There was a striking parallel between the effects of different phospholipids in the monolayer system and their effects on the overall enzyme reaction in aqueous suspension. Films of phospholipids which were active in the transferase reaction were readily penetrated by LPS, whereas the rate and extent of penetration was markedly

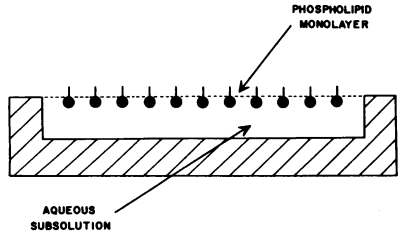


FIG. 6. *Diagram of phospholipids in a monomolecular film at an air-water interface.*

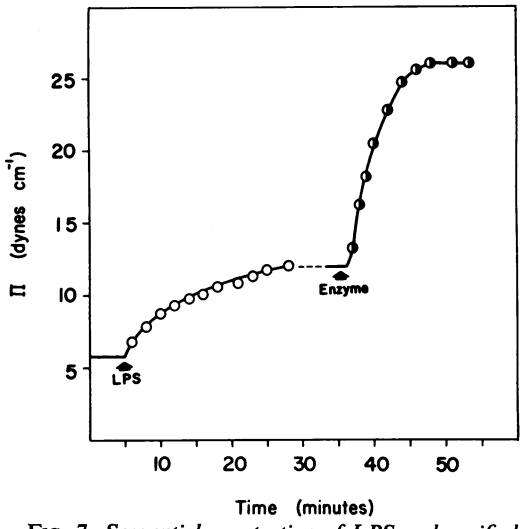


FIG. 7. *Sequential penetration of LPS and purified galactosyl transferase enzyme into a monomolecular film of PE [from Romeo et al. (59)]. A film of PE was first formed at a surface pressure (π) of 6 dynes cm^{-1}, and LPS and enzyme were injected beneath the surface (see Fig. 6).*

decreased when films of inactive phospholipids were studied (i.e., phosphatidylcholine and PE containing saturated acyl groups).

The LPS and PE molecules appear to be arranged in a side-by-side array within the binary film. The following evidence supports this arrangement. (i) The rise in surface pressure indicates an increased number of molecules on the surface. On the other hand, if LPS were adsorbed beneath a film of phospholipid, the kinetic and electrical energy of the system would be decreased, resulting in a reduction in pressure of the film (53). (ii) Changes in the acyl residues of the phospholipid caused marked changes in the ability of LPS to interact with the PE film. This implies an interaction with the fatty acyl groups of the phospholipid which are directed upward into the air phase. (iii) There was no demonstrable change in surface potential when LPS interacted with the phospholipid film. On the other hand, adsorption of molecules to the undersurface of the film would cause a significant change in potential because of the major contribution that the orientation and charge of the polar groups of the phospholipid make to the measured surface potential.

The molecular dimensions of the molecules in the plane of the film can be calculated directly, and certain inferences can be drawn about the configuration of LPS in the mixed monolayer. The calculations are based on the following assumptions. (i) The molecules in the binary film

are maximally compressed, and (ii) the orientation of PE within the mixed film is similar to its orientation in maximally compressed films of the phospholipid alone (i.e., with acyl chains directed perpendicular to the surface). The following evidence supports these assumptions. (i) The fact that the film was in a state of maximal compression was confirmed by the inability to further compress the film without expelling molecules from the surface. (ii) The lack of change in surface potential indicated that there was no significant change in orientation of the PE molecules.

A surface area of 23.2 $\pm$ 5 nm^2 per molecule of LPS was obtained in these studies, representing the area filled by each LPS molecule in the plane of the monolayer. This is close to the surface area of 25 nm^2 predicted from the known fatty acid composition of the LPS if the acyl groups are directed perpendicular to the surface. The results lend support to the idea that LPS molecules are arranged in the film with their fatty acid residues directed perpendicular to the surface. A similar orientation of LPS molecules in the absence of phospholipid has been suggested on the basis of electron microscopic (67) and X-ray studies (6) of aqueous suspensions of LPS. Figure 8 illustrates the conformation of LPS that seems most consistent with the available data. The fatty acyl residues of Lipid A are directed upward, giving a surface area of 25 nm^2 per molecule of LPS, and the polar groups are directed toward the aqueous environment. Phospholipid molecules are viewed as being interdigitated between molecules of LPS, thereby maximizing the opportunity for interactions between LPS and phospholipid. Approximately 5 to 10 molecules of PE can interact with each molecule of LPS (although only one is shown in the figure), and the distance between adjacent acyl chains is approximately 0.2 nm, small enough to permit van derWaals interactions to occur between the CH_2 groups of adjacent chains. These interactions are probably primarily responsible for the stability of the structure since the presence of high concentrations of salt in the subphase, which would tend to diminish polar group interactions, had no effect on the monolayer system or on formation of the binary complex in aqueous suspension (Fig. 4).

Incorporation of the purified transferase enzyme into the LPS-PE monolayer was the next step in the reconstitution sequence. The binary monolayer was moved to the surface of a fresh subsolution and enzyme was injected beneath the film. Penetration was indicated by a prompt rise in surface pressure (Fig. 7) and was confirmed by direct analysis of the film.

In all experiments of this type it is essential to show that function is restored to ensure that the results are relevant to the native system. This was done by injecting UDP-^{3}H-galactose into the aqueous subsolution beneath the ternary film and demonstrating that ^{3}H-galactose was then rapidly transferred into the film. Since no transfer was seen unless all three components were present, the criteria for reconstitution of the complete system were met.

Several properties of the enzyme within the film were similar to its properties in the membrane or when measured in the reconstituted system isolated by gradient centrifugation and studied in aqueous suspension. Thus, the K_m for UDP-galactose was 3.9 $\times$ 10^{-5} M for the enzyme·LPS·PE film, 6.2 $\times$ 10^{-5} M for the membrane-bound enzyme (74), and 3 $\times$ 10^{-5} M for the studies in aqueous suspension (16). Similarly, the turnover numbers of the enzyme were also similar, 0.95 and 1.04 moles of galactose per mole of enzyme per min in the film and in aqueous suspension, respectively. (Turnover number in the intact membrane cannot be determined until the number of enzyme molecules in the intact membrane is known.)

On the other hand, there was a marked difference in the total number of galactosyl residues transferred by each mole of enzyme. At completion of the reaction, 1.9 moles of galactose had been transferred for each mole of enzyme in the monolayer, and a similar ratio was obtained when the amount of enzyme in the film was reduced by 80%. In contrast, in aqueous suspension the yield is not related to the amount of enzyme, and transfer of several hundred moles of galactose per mole of enzyme is observed when small amounts of enzyme are used. This difference appears to reflect a fundamental difference between the two systems since it has been shown that the enzyme is not inactivated within the monolayer and is not lost from the film during the course of the reaction.

It is likely that each molecule of enzyme remains fixed at its original binding site in the film, perhaps catalyzing transfer only to the polysaccharide chains of a single LPS molecule or to immediately adjacent molecules. In this view, the enzyme protein would remain as a structural element of the system after completion of the reaction. The greater yield per mole of enzyme when the enzyme is studied in aqueous suspension is probably due to the presence in the aqueous system of many particles containing LPS and phospholipid (63), thereby permitting multiple collisions to occur between substrate and enzyme. On the other hand, in the monolayer (and

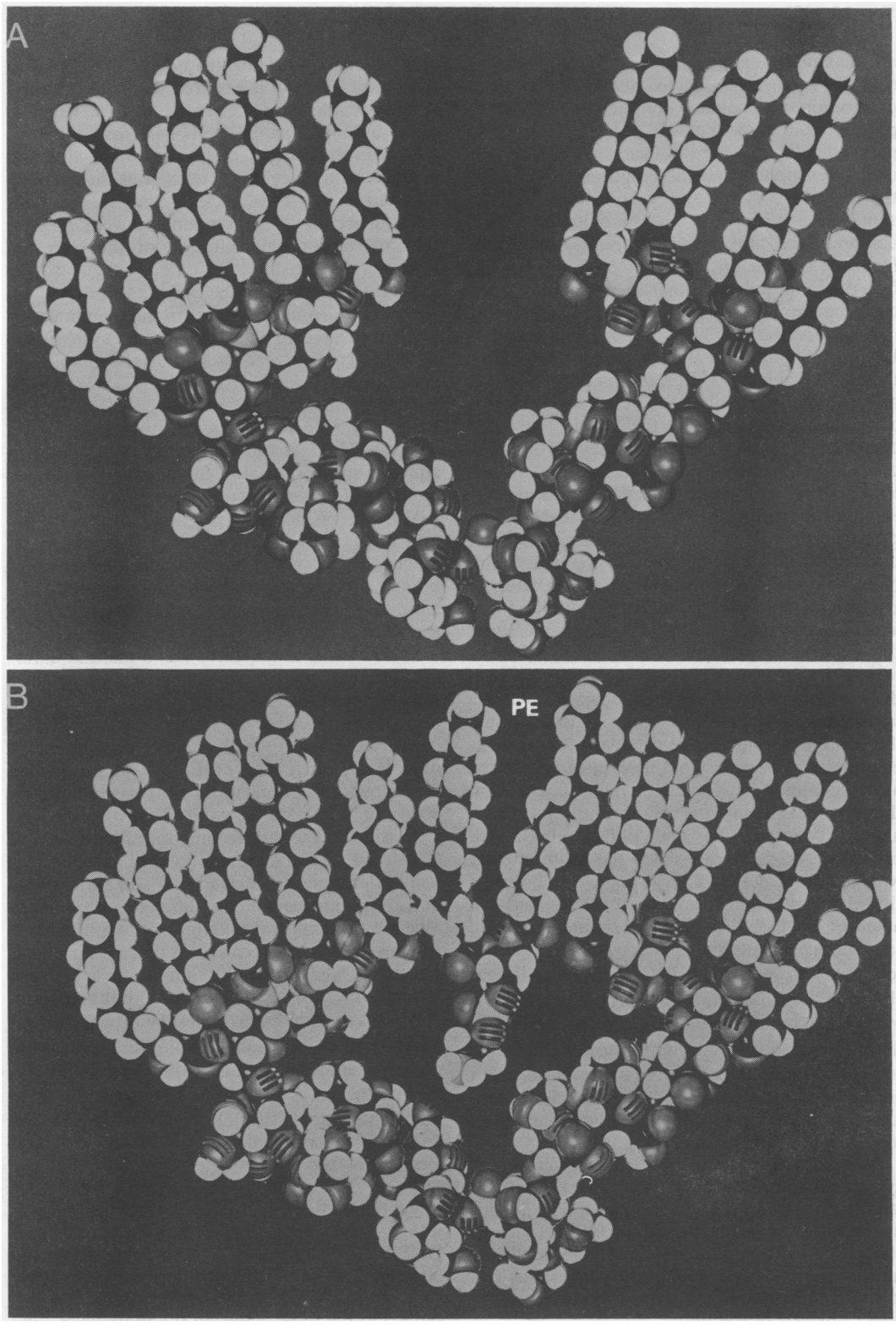


FIG. 8. *Molecular models of (A) LPS of Salmonella typhimurium G-30 (see Fig. 1), and LPS plus PE [from Romeo et al. (59)]. For clarity, only two of the three units of the LPS molecule are shown.*

possibly in the membrane as well) the components are fixed and are unable to move about after the initial interaction.

A specific sequence of addition was required for reconstitution of the system. Formation of the binary film (LPS·phospholipid) was necessary before addition of the enzyme. When the sequence was reversed (i.e., enzyme before LPS), it was impossible to form a ternary film since the presence of enzyme in the monolayer appeared to block the subsequent penetration of LPS. Although these results cannot be directly extrapolated to the almost totally unknown area of membrane biogenesis, they should be kept in mind in considering the sequence of events leading to membrane assembly in vivo.

It is likely that the arrangement of molecules in the binary and ternary monolayers is similar to their arrangement in the cell envelope, as evidenced by the marked similarities in behavior of the enzyme system in the two situations. Since the monolayer is equivalent to one-half a molecular bilayer, the results give support to the idea that the corresponding region of the membrane is basically a bimolecular leaflet.

At this time the orientation of enzyme molecules within the film cannot be fully defined (*see* Fig. 5). A portion of the molecule is certainly present in the polar region of the monolayer since the enzyme successfully catalyzes the transfer of galactose from UDP-galactose in the subphase to the distal end of the polysaccharide chains which are located in the polar region of the film.

The inhibitory effects of different LPS on the galactosyl transferase reaction suggest that part of the enzyme probably also extends deep into the core region of the polysaccharide. Lipopolysaccharides containing the inner core region acted as competitive inhibitors of the reaction (from strains LT-2, TV119, and SL1032; Fig. 1), whereas heptose-deficient LPS from mutants defective in core biosynthesis (strains G-30G and G-30 F; Fig. 1) did not inhibit the reaction (16). In the case of the inhibitory LPS, inhibition was also seen with the lipid-free LPS portion of the molecule. Therefore, the inhibition probably represents competition for a polysaccharide-binding site on the enzyme and implies that the heptose-containing backbone is involved in the enzyme-LPS interaction.

It is not known whether a portion of the enzyme molecule also extends into the nonpolar region of the film.

Some Speculations

If the behavior of the galactosyl transferase in the monolayer system is a fair representation of its behavior in the cell, the results suggest that the enzyme may be relatively fixed in position in the lipid matrix of the membrane and unable to seek new substrate molecules after completion of the transfer reaction. On the other hand, the number of transferase molecules in the native membrane is almost certainly too low to account for the normal rate of LPS synthesis during growth if each enzyme molecule catalyzes transfer to only one or two polysaccharide chains, as appears to be the case in the enzyme·LPS·PE film. (Based on average analyses, there are approximately 15 to 50 nmoles of LPS per mg of cell envelope in *S. typhimurium* G-30. If each molecule of galactosyl transferase catalyzed transfers to only one molecule of LPS, the galactosyl transferase enzyme would account for 30 to 100% of the total protein of the cell envelope.)

A model to describe the biosynthesis of the LPS core can be proposed that is consistent with these observations and with the other evidence now available. Like all such schemes based on indirect evidence, the model is speculative and may well require significant modification as further experimental results are obtained.

The model proposes that the series of glycosyl transferase enzymes that catalyze the sequential addition of sugar residues during biosynthesis of the core region of the LPS are fixed in position within the membrane (Fig. 9). In contrast, the LPS molecule is viewed as relatively mobile, moving progressively from one enzyme to the next as the polysaccharide chain is elongated. This mobility is ascribed primarily to the relatively fluid nature of the hydrocarbon residues of Lipid A, which are dissolved in the fluid hydrocarbon interior of the membrane (*see* Fig. 8), permitting the LPS to move within the membrane structure unless anchored in the polar region by an appropriate transferase enzyme. As each sugar is added, the polysaccharide chain is increased in length by one residue, thereby becoming a substrate for the next enzyme in the sequence. The nascent chain then moves to the next transferase, permitting a new LPS molecule of the proper chain length to advance to take its place. Thus, the driving force for the movement of the LPS is the affinity of the newly formed substrate for the next enzyme in the sequence. In simplistic terms, LPS molecule is pulled along the chain by the series of transferase enzymes, growing by one residue at each step. In this way each molecule of enzyme can catalyze the transfer of glycosyl residues to a large number of acceptor molecules while remaining in a relatively fixed position within the membrane. As indicated in Fig. 9, each enzyme molecule is associated with a

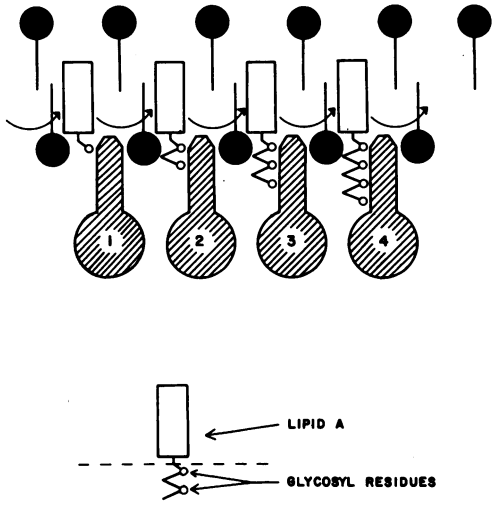


FIG. 9. *Speculative model of the organization of the machinery for biosynthesis of the LPS core. Symbols are as described in Fig. 5. Enzymes 1, 2, 3, and 4 represent four sequential glycosyl transferases (e.g., the enzymes transferring glucose, galactose, glucose, and N-acetylglucosamine, respectively, to the inner core as shown in Fig. 1).*

nascent chain of the proper length and is unavailable to other LPS molecules until the reaction is complete and the polysaccharide chain has been removed.

The terminal portion of the polysaccharide chain is presumed to be the major site of binding to the enzyme since the terminal sugar residue is the acceptor site for the enzyme-catalyzed transfer reactions. In addition, as mentioned above, competition experiments suggest that the heptose-containing inner core region also interacts with the transferase enzyme. Unless the next transferase is available, this secondary interaction may be sufficient to maintain the association of polysaccharide and transferase enzyme even after the reaction has been completed. In the monolayer experiments, the next enzyme was not present and therefore the lipopolysaccharide molecule did not leave the galactosyl transferase enzyme. It is possible that decreased mobility of the LPS in the monolayer as opposed to the membrane may also play a role.

The model predicts that the transferase enzymes are arranged in the proper sequence and are located adjacent or in close proximity to each other, thereby facilitating the efficient and ordered growth of the polysaccharide chains without requiring excessive movement of LPS molecules within the membrane structure. The enzymes of O-antigen synthesis (*see* Glycosyl Carrier Lipid in O-Antigen Synthesis) could be organized in a similar manner.

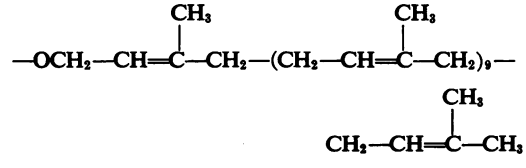
Although most of the cellular LPS is located in the outer membrane of the cell envelope, it is likely that biosynthesis of the core region takes place in the cytoplasmic membrane (M. J. Osborn, *personal communication*). If this is so, a transport mechanism will be required to move the completed lipopolysaccharide to the outer membrane, and a transport molecule (or molecules) might complete the sequence by pulling the completed LPS from the last enzyme, thereby permitting the chain of reactions to continue.

GLYCOSYL CARRIER LIPIDS

Identification of Carrier Lipids

In 1965, Anderson et al. (1) discovered that lipid-linked intermediates are involved in biosynthesis of the peptidoglycan of *Staphylococcus aureus*, and this was soon followed by the demonstration in the laboratories of Weiner et al. (79) and Wright et al. (84) that similar lipid-linked intermediates participate in biosynthesis of the O-antigens of *Salmonella*. This led to the description of a new mechanism of macromolecular synthesis, in which the intermediates are covalently linked to a "carrier" lipid. The carrier lipid has been variously called antigen carrier lipid (83) and glycosyl carrier lipid (61). The term glycosyl carrier lipid (GCL) seems preferable since it is applicable to systems other than the O-antigen system; therefore, this terminology will be used throughout this review.

The structure of the carrier lipids in the peptidoglycan and O-antigen systems were first established by Higashi and his colleagues (21) and by Wright and his collaborators (83), primarily by the use of mass spectrometry. Since then, the identity of carrier lipids in several other systems has also been established and in all cases the lipid was shown to be a derivative of a C_{55} polyisoprenoid alcohol:



These compounds are present in the cell envelope in very small amounts and act catalytically in the biosynthesis of a variety of cell envelope polymers. The lipid-linked intermediates in the reactions are of two types, both involving linkage of the reducing end of a sugar or oligo-

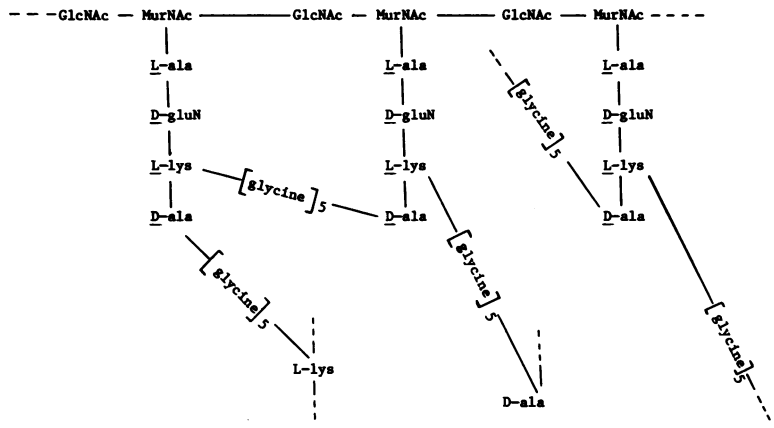
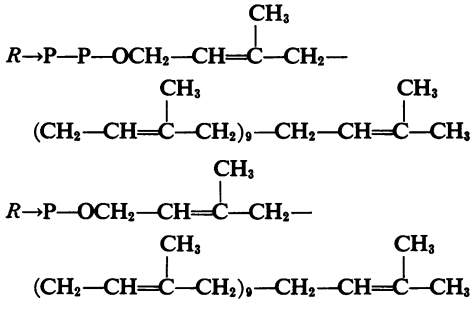
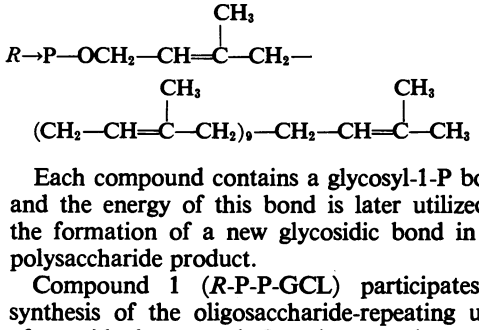


FIG. 10. *Structure of the peptidoglycan of Staphylococcus aureus strain Copenhagen. The pentaglycine cross-bridges are meant to connect peptide units in different polysaccharide chains as well as within the same chain.*

saccharide residue (*R*) to the hydroxyl group of the lipid via a phosphate or pyrophosphate group (compounds 1 and 2).



(1)



(2)

Each compound contains a glycosyl-1-P bond, and the energy of this bond is later utilized in the formation of a new glycosidic bond in the polysaccharide product.

Compound 1 (*R*-P-P-GCL) participates in synthesis of the oligosaccharide-repeating units of peptidoglycan and O-antigen, and may be involved in the biosynthesis of other polymers which also contain large numbers of repeating oligosaccharide units. Compound 2 (*R*-P-GCL) has been identified as an intermediate both in mannolipid biosynthesis (70) and in enzymatic reactions leading to modification of O-antigen chains (81). It may, therefore, be the preferred intermediate when only a single sugar residue or a small number of residues must be transferred.

Glycosyl Carrier Lipids in Peptidoglycan Synthesis

Peptidoglycan structure and biosynthesis. The structure of the peptidoglycan of *Staphylococcus aureus* is shown in Fig. 10. The same general polysaccharide backbone is found in the peptidoglycan of other organisms, but various modifications of the peptide portions of the molecule can occur. Some of these are discussed below.

An understanding of the mechanism of synthesis of peptidoglycan has emerged from studies in several laboratories over the past decade, most notably from the work of Strominger and his colleagues, and the general sequence of reactions appears to be similar in most organisms. The biosynthetic sequence can be divided arbitrarily into four stages: (i) formation of nucleoside phosphate derivatives of muramic acid and glucosamine (UDP-MurNAc-pentapeptide and UDP-GlcNAc), (ii) formation of disaccharide units linked to a carrier lipid, (iii) transfer from carrier lipid to the linear polysaccharide backbone, and (iv) closure of peptide cross-links (transpeptidation).

(i) Lipid intermediates are not involved in the first series of reactions, which lead to synthesis of UDP-MurNAc-pentapeptide and UDP-GlcNAc. It should be noted that activation of the acetylmuramyl and acetylglucosaminyl residues occurs during this stage with formation of the high energy MurNAc-1-P and GlcNAc-1-P bonds in the nucleoside diphospho compounds. The energy of the acetylmuramyl-1-P bond is conserved throughout the remainder of the biosynthetic sequence and is finally utilized in formation of a new glycosidic bond during the polymerization reaction.

(ii) Although it had been suspected for many years that UDP-MurNAc-pentapeptide and UDP-GlcNAc were the precursors of the polysaccharide backbone of peptidoglycan, it was not

until 1964 that this was directly demonstrated. In that year Chatterjee and Park (12) and Meadow et al. (37) showed that fragments of cell wall from *S. aureus* contained enzymes that catalyze the transfer of MurNAc-pentapeptide and GlcNAc from their UDP derivatives into the endogenous peptidoglycan of the cell wall fragments. This was no surprise since nucleoside diphosphate sugars were known to act as glycosyl donors in the synthesis of many other polysaccharides and since UDP-MurNAc-pentapeptide and UDP-GlcNAc were known to be present in extracts of *S. aureus* (51). However, the system differed from other polysaccharide-synthesizing systems in the products of the reaction. In all other systems, the nucleotide products of the reactions had been the nucleoside diphosphates (e.g., UDP-glucose + glycogen → glucosyl-glycogen + UDP).

In contrast, the peptidoglycan-synthesizing system resulted in formation of both UDP and uridine monophosphate (UMP). The UDP originated from UDP-GlcNAc, whereas the UMP was entirely derived from the UDP-MurNAc-pentapeptide. This was shown by specific radioactive labeling experiments (1) and by demonstrating that radioactive UMP exchanged with UDP-MurNAc-pentapeptide (76). The discovery of the exchange reaction led Struve and Neuhaus (76) to suggest that MurNAc-pentapeptide was first transferred to an intermediate acceptor and that the polymerization with GlcNAc occurred at a later step.

$$\text{UDP-MurNAcMpentapeptide} + \text{acceptor} \rightleftharpoons \text{acceptor-P-MurNAc-pentapeptide} + \text{UMP} \quad \text{VI}$$

The occurrence of the exchange reaction also revealed another difference between peptidoglycan synthesis and synthesis of other polysaccharides, namely, the reversibility of the nucleotide sugar transferase step. This is a reflection of the conservation of the high energy of the glycosyl-1-P bond in the product of the reaction. In contrast, the usual glycosyl transferase reactions (i.e., UDP-sugar + acceptor → glycosyl-acceptor + UDP) are essentially irreversible because of formation of the more stable glycosidic bond in the glycosyl-acceptor product.

Direct evidence for an intermediate carrier was obtained by Anderson et al. (1) who isolated the product of reaction VI and demonstrated the lipid-soluble nature of the acceptor (MurNAc-pentapeptide-P-P-GCL; reaction 1 in Fig. 11). These studies also showed that GlcNAc was subsequently transferred directly to the lipid-linked intermediate to form the disaccharide unit (GlcNAc-MurNAc) which later appears in peptidoglycan. The transfer of GlcNAc from UDP-GlcNAc to MurNAc-pentapeptide-PP-GCL (reaction 2 in Fig. 11) is similar to other glycosyl transferase reactions, resulting in formation of a new glycosidic bond with elimination of UDP. Evidence that the UDP originated solely from UDP-GlcNAc was obtained by radioactive labeling experiments. When the reaction mixture contained UD^{32}P-GlcNAc and nonradioactive UDP-MurNAc-pentapeptide, the ^{32}P appeared in UDP but not in UMP. Conversely UD^{32}P-MurNAc-pentapeptide gave rise to UM^{32}P and not to UD^{32}P. It should be noted that the disaccharide product of the reaction (GlcNAc-MurNAc-pentapeptide) is still linked to GCL via the muramyl-1-P group.

Further modifications of the peptide take place while the disaccharide-pentapeptide is still linked to the carrier lipid. The detailed structure of the peptide portion of the peptidoglycan is quite variable in different bacterial strains, and various modification reactions can occur. In *S. aureus*, the major modification involves addition of the pentaglycine chain that later serves to cross-link the peptides within the peptidoglycan structure (*see* Fig. 10). Matsuhashi et al. (36) showed that glycyl-transfer RNA (tRNA) serves as the glycyl donor in these reactions (reaction 3 in Fig. 11). This was the first demonstration of a role for amino acyl-tRNA in a process other than protein synthesis. There is no evidence that ribosomes or messenger RNA participate in formation of the pentaglycine chain, but detailed study of the reaction has been hampered by the difficulties of working with a particulate enzyme system.

Comparable modification reactions have been shown to occur in other organisms. These include the incorporation of L-serine and glycine in *S. epidermidis* (52), L-alanine in *Arthrobacter crystallopoietes* (56), and L-threonine in *Micrococcus roseus* (57). In all cases, aminoacyl-tRNA is the amino acyl donor. Incorporation of D-aspartic acid by enzyme preparations from *Streptococcus fecalis* and *Lactobacillus casei* has also been reported, but these reactions do not appear to involve tRNA (75).

Modifications of the D-glutamyl residue of the peptide also occur at the level of the disaccharide-PP-GCL. These include amidation in *Staphylococcus aureus* (73) and addition of a single glycine residue to the α-carboxyl group in *M. lysodeikticus* (25).

(iii) Finally, when the modification reactions

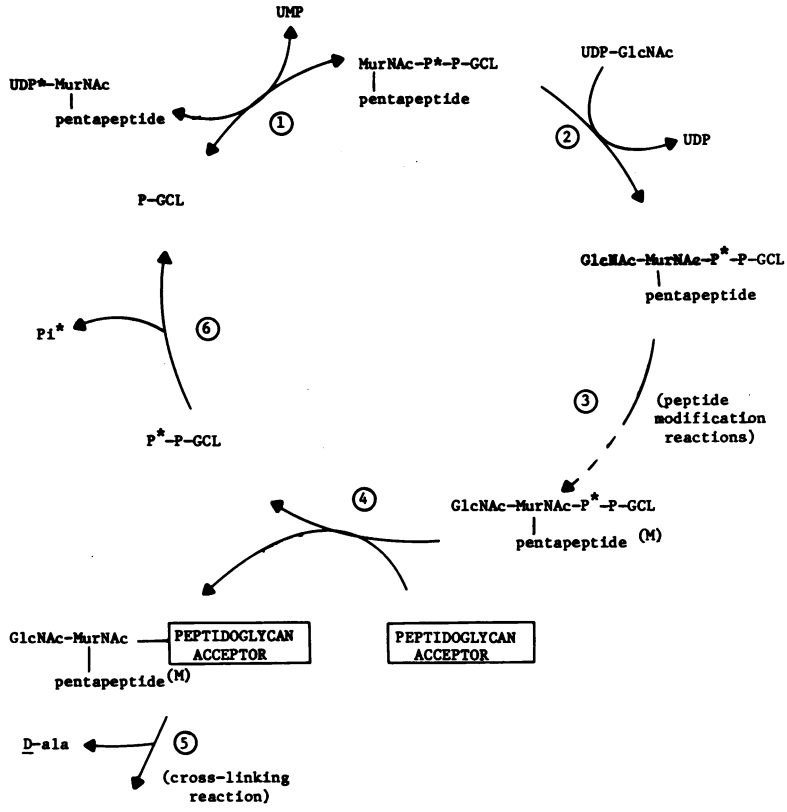


FIG. 11. *Sequence of reactions leading to synthesis of peptidoglycan. Pentapeptide (M) represents the pentapeptide after the various modification reactions (i.e., in Staphylococcus aureus, after amidation of the γ-D-glutamyl residue and addition of the pentaglycine chain to the L-lysine residue in the Pentapeptide). P* represents the phosphate group that originates from UDP-Mur-NAc-pentapeptide.*

have been completed the disaccharide-peptide is transferred from carrier lipid to the endogenous peptidoglycan of the cell wall, resulting in elongation of the polysaccharide backbone of the peptidoglycan and release of the lipid (reaction 4 in Fig. 11).

(iv) At this point, an additional reaction is still required to cross-link the peptide chains within the peptidoglycan (reaction 5 in Fig. 11). This cross-linking reaction does not involve the carrier lipid, which has been released in the preceding step. In *E. coli* at least, the cross-linking reaction takes place by a transpeptidation mechanism leading to release of the terminal D-alanyl residue of a peptide unit in the peptidoglycan (22). This reaction is inhibited by penicillin and appears to represent the primary site of action of the antibiotic.

Structure of the carrier lipid in peptidoglycan synthesis. The carrier lipid of *M. lysodeikticus* has been isolated and its structure has been determined (21; *see* compound 1 in Identification of Carrier Lipids). The polyisoprenoid alcohol portion of the lipid is linked to the glycosyl residues via a pyrophosphate bridge, and therefore lipid pyrophosphate (PP-GCL) is released when the oligosaccharide unit is finally transferred to the peptidoglycan acceptor. This compound must be further modified by release of Pi (reaction 6, Fig. 11) before it can participate in another turn of the biosynthetic cycle since the initial reaction (reaction 1, Fig. 11) utilizes GCL-monophosphate (P-GCL).

Glycosyl Carrier Lipid in O-Antigen Biosynthesis

Mechanism of biosynthesis of O-antigen. Shortly after the discovery that a carrier lipid was involved in peptidoglycan synthesis, it was shown that a very similar mechanism operates in biosynthesis of the O-antigens of *Salmonella* (79, 84). There is a formal similarity in the basic structures of bacterial peptidoglycans and O-antigens because both are composed of repeating sequences of oligosaccharide units and

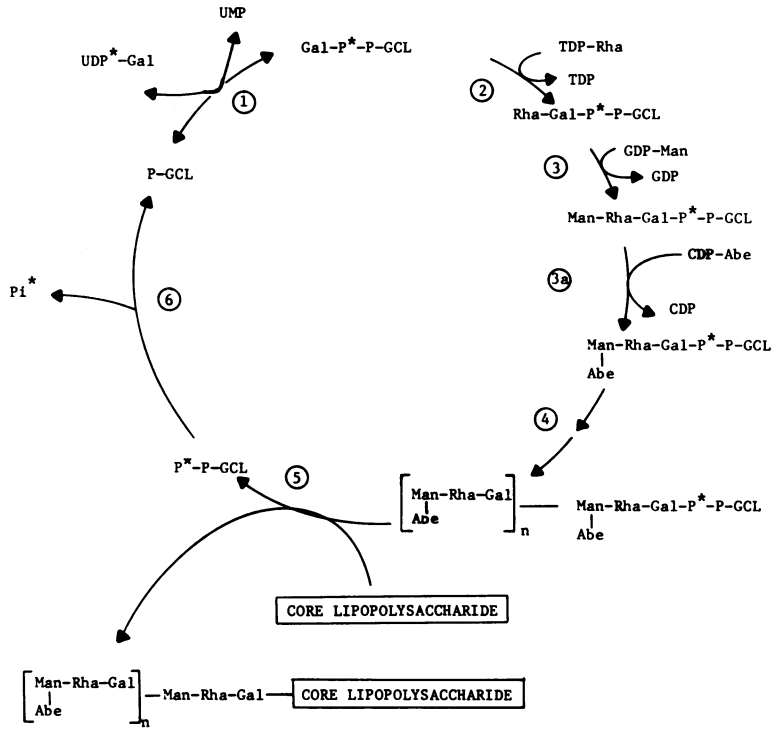


FIG. 12. *Sequence of reactions leading to synthesis of O-antigen in Salmonella typhimurium. P* represents the phosphate group that originates from UDP-Gal.*

in both cases the oligosaccharide units are synthesized by a GCL-linked series of reactions. In the case of the O-antigens, however, both the formation of the individual oligosaccharide units *and* their later polymerization take place while linked to the carrier lipid. In the final step, the complete O-antigen is transferred to the core region of the lipopolysaccharide molecule. Therefore, the pathway of biosynthesis can be divided into three parts: (i) synthesis of oligosaccharide-lipid in reactions catalyzed by a series of *transferases*; (ii) polymerization of oligosaccharide units [oligosaccharide-lipid → (*oligosaccharide*)-lipid] catalyzed by O-antigen polymerases; and (iii) transfer of the polysaccharide chain to the LPS acceptor catalyzed by O-antigen:LPS *ligases*. [The enzyme catalyzing this step has been called both translocase (31) and ligase (45). It was suggested by Osborn (45) that the enzyme should be called O-antigen:LPS ligase to avoid confusion with the peptidoglycan system in which the term translocase has been applied to the *first* step in the biosynthetic cycle.]

The pathway is essentially identical in *Salmonella typhimurium*(79) and *S. newington* (84). The O-antigens of both species contain a trisaccharide repeating unit, Man-Rha-Gal (Fig. 1), and synthesis of the trisaccharide occurs in a manner analogous to synthesis of the disaccharide unit (GlcNAc-MurNAc-pentapeptide) of peptidoglycan.

(i) In the first reaction, Gal-1-P is transferred from UDP-galactose to the carrier lipid with formation of Gal-1-P-P-GCL (reaction 1, Fig. 12); UMP is released and the high energy of the Gal-1-P bond of the nucleotide sugar is conserved. This initial reaction is freely reversible with an equilibrium constant of approximately 0.5 (48). The remaining steps in synthesis of the trisaccharide include the sequential transfer of rhamnosyl and mannosyl residues from thymidine diphosphate (TDP)-rhamnose and GDP-mannose with formation of the complete trisaccharide, still linked to the carrier lipid by a pyrophosphate group [Rha-Man-Gal-1-P-P-GCL (reactions 2 and 3, Fig. 12)]. It is assumed that the nucleoside diphosphates TDP and GDP are released in these steps, and the reactions are believed to be not reversible because of the formation of the two stable glycosidic bonds. In *S. typhimurium* the trisaccharide is further modified by addition of abequose (3,6-dideoxy-D-galactose) from CDP-abequose (49), resulting in formation of the tetrasaccharide unit of the

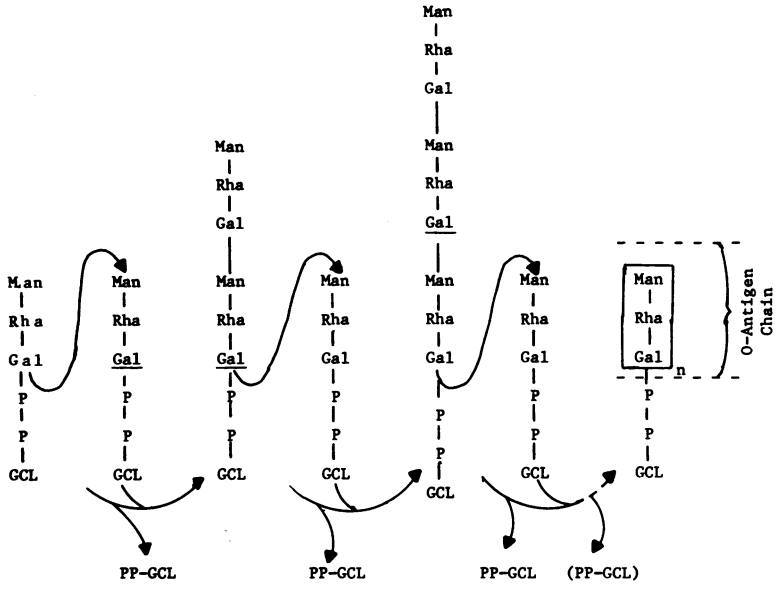


FIG. 13. *Mechanism of chain elongation in the O-antigen polymerization reaction in Salmonella newington [from Bray and Robbins (4a)].*

S. typhimurium O-antigen, still linked to the carrier lipid (reaction 3a, Fig. 12), whereas in *S. newington* the trisaccharide is polymerized without further modification (84).

(ii) The individual oligosaccharide units are then polymerized to form the complete O-antigen chain, still linked to the carrier lipid (reaction 4, Fig. 12). The mechanism of the polymerization reaction in *S. newington* has been studied in detail by Bray and Robbins (4a) who showed that growth occurs at the reducing end of the polymer. The polymerization occurs by a series of stepwise transfers of the growing polysaccharide chain to the mannosyl residue of trisaccharide-P-P-GCL units (Fig. 13). It is therefore analogous to the growth of proteins, in which the incomplete peptide is transferred to the next amino acid in the sequence, and is not similar to the synthesis of glycogen in which individual sugar residues are added to the *nonreducing* end of the growing polysaccharide chain.

(iii) Finally, the fully polymerized O-antigen is transferred to the core region of the LPS acceptor with release of P-P-GCL (reaction 5, Fig. 12). The site of attachment in the core has not yet been determined for the enzymatically synthesized product. However, Nikaido (41) recently showed that the O-antigen chains of *S. typhimurium* are normally linked to the distal glucosyl residue of the core region (Fig. 1); therefore, it is likely that the O-polysaccharide is transferred directly to this residue after completion of the polymerization reactions.

All of the reactions described above have been demonstrated in cell-free extracts including formation of oligosaccharide-P-P-GCL, polymerization of oligosaccharide units to form $(\text{oligosaccharide})_n$-P-P-GCL, and the ligase reaction which results in formation of the complete *Salmonella* LPS. The enzyme activities are membrane-bound, together with the carrier lipid, and it is likely that all of the reactions described in Fig. 12 occur in the membrane. It has not yet been established whether the reactions take place in the cytoplasmic membrane or in the outer membrane of the *Salmonella* cell envelope. There is now considerable evidence (38, 50, 66, 71) that most of the lipopolysaccharide of the cell is located in the outer membrane. Thus, if O-antigen or oligosaccharide-P-P-GCL synthesis occurs in the cytoplasmic membrane, a transfer mechanism must exist to transport either $(\text{oligosaccharide})_n$-P-P-GCL or completed LPS to the outer membrane. On the other hand, the reactions require a constant supply of nucleotide sugars which are presumably located in the cytoplasm. Therefore, if synthesis of O-antigen occurs in the outer membrane, the cell requires a mechanism to bring nucleotide sugars across the cytoplasmic membrane. In either case, the answer will be of great interest.

Mutants in O-antigen biosynthesis. Among the general class of rough mutants of *Salmonella*, several strains are known which are blocked either in the ability to synthesize O-antigen or

to attach the O-antigen to the core lipopolysaccharide (34). These mutants fall in four classes: (i) mutants unable to synthesize the trisaccharide repeating unit, (ii) mutants unable to synthesize the complete core region of the RII LPS (Fig. 1), (iii) mutants with defective O-antigen polymerase, and (iv) mutants with defective O-antigen: LPS ligase.

(i) This class includes mutants deficient in synthesis of the nucleotide sugar precursors of the O-antigen (UDP-galactose, TDP-rhamnose, GDP-mannose, CDP-abequose). These strains are obviously unable to form oligosaccharide-PP-GCL. Within this group, strains deficient in synthesis of UDP-galactose, TDP-rhamnose GDP-mannose, and CDP-abequose have been described, and the LPS of all of these strains lack the O-antigen (42*a*, 42*b*, 43, 60). One would also predict the occurrence of mutants defective in one of the transferase enzymes involved in assembly of oligosaccharide-P-P-GCL (steps 1 to 3a in Fig. 12), but these have not yet been described.

(ii) Since the lipopolysaccharides of these mutants lack the O-antigen attachment site they cannot transfer the O-polysaccharide from (oligosaccharide)$_n$-P-P-GCL to the LPS acceptor. Thus far, these include strains deficient in the following core glycosyl transferase enzymes (*see* Fig. 1): UDP-glucose:LPS glucosyl transferase I (44), UDP-galactose:LPS α,3-galactosyl transferase (44), and probably UDP-*N*-acetylglucosamine:LPS *N*-acetylglucosaminyl transferase (2). In all cases, the core region of the LPS is incomplete. Although their LPS lack the O-antigen, the cell envelopes of these organisms appear to contain (oligosaccharide)$_n$-P-P-GCL which accumulates in the membrane because of the lack of a suitable LPS acceptor. When whole cells of *rfa* strains are treated with aqueous phenol, a water-soluble polymer (O-specific hapten) is formed (2). The polymer exhibits the O-antigenic specificity and contains all of the O-specific sugars but lacks the components of the core and lipid A portions of the wild-type LPS. The studies of Kent and Osborn (26) provided evidence that the structure of the O-specific hapten was (oligosaccharide)$_n$-P and that it appeared to originate from (oligosaccharide)$_n$-P-P-GCL in the cell envelope by hydrolytic cleavage of the labile pyrophosphate bond during the extraction procedure. The number of moles of O-specific hapten was approximately equal to the total number of moles of GCL in the cell envelope. The pyrophosphate bond of the parent compound is extremely susceptible to hydrolysis, and this presumably explains the failure thus far to isolate the complete lipid-linked O-antigen (oligosaccharide$_n$-PP-GCL) which is thought to accumulate in these organisms (26).

TABLE 1. *O-antigens of Salmonella anatum*

Organism	Repeating unit of O-antigen	Antigen[a]
Nonlysogenic	(Man-Rha-GalOAc[b] $\xrightarrow{\alpha}$)	O_{10}
Lysogenic for ϵ^{15}	(Man-Rha-Gal $\xrightarrow{\beta}$)	O_{15}
Lysogenic for ϵ^{15} and ϵ^{34}	(Man-Rha-Gal $\xrightarrow{\beta}$) $\uparrow\alpha$ Glc	$O_{15,34}$
Lysogenic for ϵ^{34}	(Man-Rha-GalOAc $\xrightarrow{\alpha}$)	O_{10}

[a] Lipopolysaccharides of these organisms also have antigen 3 specificity (O_3).
[b] GalOAc, O-acetyl-D-galactosyl.

Mutants unable to synthesize one of the nucleotide sugar precursors of the core polysaccharide also fall into this general class. In some of these cases, however, a sugar may appear both in the core and in the O-antigen, as in the case of D-galactose in *S. typhimurium* in which UDP-galactose is the precursor of the galactosyl residues of both core and O-antigen. In these cases, synthesis of the oligosaccharide units of the O-antigen is not possible and the incomplete core structure is not the sole cause of the lack of O-antigen. Therefore, in UDP-galactose-deficient mutants of *S. typhimurium*, for example, "O-specific hapten" does not accumulate.

(iii) Studies of *S. anatum* and of bacteriophage ϵ^{15} by Robbins and his collaborators have demonstrated the effects of genetic defects in the O-antigen polymerase. The phage contains the genetic information for a new O-antigen polymerase (O_{15} polymerase) and infection of group E *Salmonella* with the virus results, therefore, in formation of a new O-antigen (antigen 15) in the infected cell (*see* Table 1). Simultaneously, the endogenous polymerase activity (O_{10} polymerase) of the host is inhibited. A temperature-sensitive mutant of ϵ^{15} was studied by Bray and Robbins (4) and was shown to produce a temperature-sensitive O-antigen polymerase (O_{15} polymerase^{ts}). Cells lysogenic for the mutant phage were able to synthesize Man-Rha-Gal-P-P-GCL at elevated temperature but were unable to polymerize this product to (Man-Rha-Gal)$_n$-PP-GCL. A comparable temperature-sensitive bacterial mutant of *S. anatum* has also been described (31). The O-antigen polymerase activity of this organism (O_{10} polymerase^{ts}) was shown to be markedly decreased at elevated temperatures, whereas synthesis of Man-Rha-

Gal-PP-GCL was unaffected. A semirough mutant (*rfc*) of *S. typhimurium* also appears to fall in this general class (39).

(iv) A fourth class of O-antigen mutants should also exist, deficient in the ligase which catalyzes transfer of O-antigen from $(\text{oligosaccharide})_n$-PP-GCL to LPS core. These mutants should accumulate O-specific hapten because of the ligase deficiency but should contain a complete LPS core, thereby differing from organisms in group iii. Strains with this phenotype are known (18), but thus far characterization of the enzymatic defect has not been reported.

Structure of the carrier lipid in O-antigen biosynthesis. The structure of the carrier lipid in the O-antigen pathway was determined by the analysis of degradation products of Rha-Gal-P-P-GCL and Gal-P-P-GCL. Mild acid hydrolysis was used to hydrolyze Rha-Gal-P-P-GCL to Rha-Gal + PPi + lipid. Mass spectrometry revealed that the lipid contained 11 isoprene units, each unit containing one double bond, and inorganic pyrophosphatase was used to identify PPi (83). The oligosaccharide-1-P linkage was established in both the *S. typhimurium* and *S. newington* systems by subjecting Gal-P-P-GCL and oligosaccharide-P-P-GCL to mild alkaline hydrolysis. This resulted in formation of oligosaccharide cyclic phosphate (R-Gal-1,2 cyclic phosphate) and P-GCL (79, 84). Thus, the carrier lipid and the structure of the oligosaccharide-lipid intermediates are essentially identical in the O-antigen and peptidoglycan systems.

Glycosyl Carrier Lipid in Mannolipid Biosynthesis

A carrier lipid mechanism is also involved in biosynthesis of a mannan located in the cell membrane fraction of *M. lysodeikticus*. The lipid is a C_{55} polyisoprenoid compound similar to the carrier lipids involved in synthesis of peptidoglycan and O-antigen, as shown by the essentially identical mass spectra obtained from the three lipids (27, 70). However, the mannan-synthesizing system differs in several important respects from the O-antigen and peptidoglycan systems. The major difference concerns the first step in the biosynthetic sequence.

$$\text{GDP-mannose} + \text{P-GCL} \rightleftarrows \text{Man-P-GCL} + \text{GDP} \quad \text{VII}$$

$$\text{Man-P-GCL} + \text{mannan acceptor} \rightarrow \text{Man-Mannan} + \text{P-GCL} \quad \text{VIII}$$

In the first reaction (equation VII), a mannosyl residue is transferred from GDP-mannose to P-GCL with formation of mannosyl-1-P-GCL and GDP. This differs from both the O-antigen and peptidoglycan-synthesizing systems in which a sugar-1-P residue is transferred and the products are glycosyl-P-P-GCL and the corresponding nucleoside *mono*phosphate. The reaction is reversible (70) and in this respect is similar to the first step in the other GCL-mediated reactions. The mannosyl residue is then transferred to the mannan acceptor with release of P-GCL (equation VIII). This contrasts with the O-antigen and peptidoglycan systems in which additional sugars are added while the initial glycosyl residue is still linked to the carrier lipid.

The mannan is a homopolymer containing a mixture of $1 \rightarrow 2$, $1 \rightarrow 3$, and $1 \rightarrow 6$ mannosidic linkages with a small amount of branching. Methylation studies by Sher and Lennarz (69) showed that the mannosyl residue is transferred from Man-P-GCL primarily to the nonreducing terminus of the acceptor mannan. The specific radioactivity incorporated from ^{14}C-Man-P-GCL was much higher in 2,3,4,6-tetra-O-methyl-mannoside than in any of the trimethylmannosides identified in the product. This establishes the nonreducing terminal position of the enzymatically incorporated mannose and indicates that only a single sugar residue is transferred, as opposed to the transfer of oligosaccharide units in the peptidoglycan and O-antigen systems. Most of the ^{14}C-mannose incorporated was released as free mannose when the product was treated with α-mannosidase, thereby supporting the nonreducing terminal location of the sugar. No evidence for initiation of new mannan chains was obtained.

Bacitracin did not inhibit the mannan-synthesizing system, in contrast to its marked inhibitory effect on peptidoglycan and O-antigen synthesis. This is consistent with the proposed mode of action of the antibiotic, which appears to act by inhibiting the dephosphorylation of GCL-pyrophosphate (72):

$$\text{P—P—GCL} \xrightarrow{\text{Bacitracin}} \text{P—GCL} + \text{Pi} \quad \text{IX}$$

In the O-antigen and peptidoglycan systems (Fig. 11, 12), P-P-GCL is the end product of the biosynthetic cycle and must be dephosphorylated to permit another turn of the cycle. In contrast, the mannolipid system leads directly to the production of P-GCL; therefore, no subsequent dephosphorylation step is necessary. Thus, bacitracin has no effect.

Glycosyl Carrier Lipid in the Synthesis of Other Bacterial Polymers

Lysogenic conversion of Salmonella O-antigen. The O-antigen of *S. anatum* consists of a repeating trisaccharide unit whose structure is

modified when the organism is lysogenic for the temperature bacteriophages ϵ^{15} and ϵ^{34} (55; Table 1). Biosynthesis of the basic Man-Rha-Gal repeating unit involves a GCL mechanism and is described above (Fig. 12). Recently, however, Wright (81) demonstrated that GCL also participates in the ϵ^{34} phage-directed modification of the O-antigen. As shown in Table 1, this modification consists of the addition of single glucosyl residues as branches on the repeating sequence of β-linked trisaccharide units of the ϵ^{15} lipopolysaccharide.

Crude enzyme preparations of *S. anatum* (ϵ^{34}) or *S. anatum* (ϵ^{15}, ϵ^{34}) catalyze the transfer of glucose from UDP-glucose into glucosyl-P-GCL.

UDP-glucose + cell envelope
$\leftrightharpoons$ Glc-P-GCL + UDP X

In the single lysogen [*S. anatum* (ϵ^{34})], the Glc-P-GCL product is not transferred into O-antigen. On the other hand, in organisms lysogenic for both bacteriophages [i.e., *S. anatum* (ϵ^{15}, ϵ^{34})] the glucosyl residue is ultimately transferred into the O-antigen.

Glc-P-GCL + acceptor
$\rightarrow$ Glc-acceptor + P-GCL XI

This suggests that the glucosyl transferase enzyme (equation XI) requires the ϵ^{15} O-antigen as acceptor. The galactosyl residues in the O-antigen of the nonlysogenic organism or in the ϵ^{34} single lysogens are O-acetylated and are in α linkage; these are thought to be unsuitable acceptors for glucose transfer as evidenced by the absence of glucose in the ϵ^{34} O-antigen despite the presumed presence of the glucosyl transferase enzyme. Presumptive evidence that the glucosyl transferase enzyme (equation XI) is a product of the ϵ^{34} genome, hence present in ϵ^{34} single lysogens, has come from the isolation of mutants of ϵ^{34}. Cells infected with the mutant bacteriophage (i.e., *S. anatum* (ϵ^{15}, ϵ^{34*})) synthesize Glc-P-GCL but do not transfer the glucosyl residue to the ϵ^{15} O-antigen which is present in the cell (82) and which acts as the final glucosyl acceptor in cells containing the wild-type virus [i.e., *S. anatum* (ϵ^{15}, ϵ^{34})] as described above.

Consistent with this general hypothesis is the observation that Glc-P-GCL is the major product of the reaction catalyzed by the *S. anatum* (ϵ^{34}) preparations, whereas both Glc-P-GCL and Glc-LPS are formed when preparations from *S. anatum* (ϵ^{15}, ϵ^{34}) are studied. Evidence that Glc-P-GCL is the precursor of Glc-LPS in *S. anatum* (ϵ^{15}, ϵ^{34}) was obtained from two-step experiments with UDP-glucose-^{14}C as the glycosyl donor. During the initial 5 min of the reaction, approximately equal amounts of ^{14}C-Glc-P-GCL and ^{14}C-Glc-LPS were formed. With time, there was a significant decrease in the amount of radioactivity in ^{14}C-Glc-GCL. Thus far, however, direct transfer from added Glc-P-GCL to LPS has not been reported.

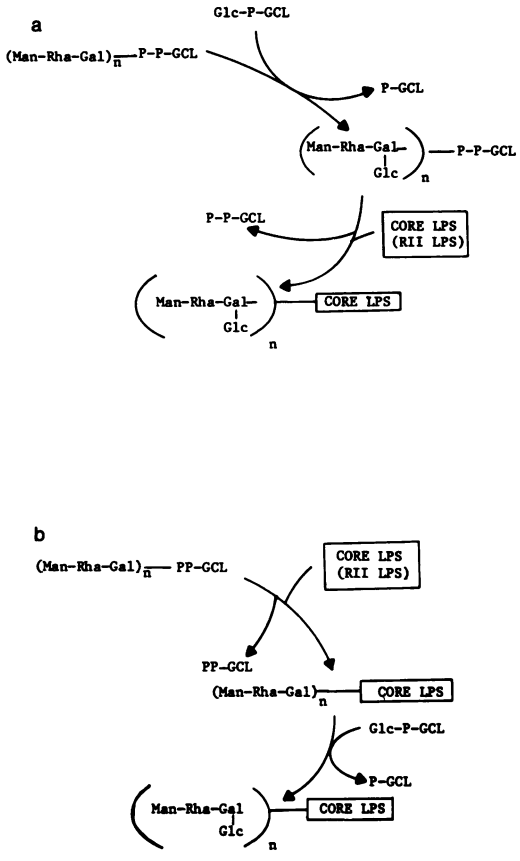


FIG. 14. *Alternative possibilities for the glucosylation of the O-antigen chain in Salmonella newington* (ϵ^{15}, ϵ^{34}).

When studied by mass spectrometry, the glucosyl carrier lipid consisted of a mixture of C_{55} and C_{50} polyisoprenoid alcohols. It is therefore closely related or identical to the carrier lipids that participate in the synthesis of peptidoglycan, mannolipid, and the repeating oligosaccharide units of the O-antigens of *Salmonella*.

The first step in the sequence of reactions (equation X) differs from the first step in synthesis of the remainder of the O-antigen since a glycosyl residue rather than a glycosyl-1-P residue is transferred from UDP-glucose. The nucleotide product is therefore UDP rather than UMP, and the reaction is similar to the initial reaction in mannolipid synthesis (*see* equation VII). Since the first step (equation X) could not be demonstrated in extracts from cells lacking

the ϵ^{34} genome, the enzyme catalyzing this initial step appears to be either a product of the ϵ^{34} genome or under the control of a phage product.

It is not clear whether the glucosyl residue is added to O-antigen-P-P-GCL (Fig. 14a) or whether glucose addition occurs after the O-antigen has been transferred to the core LPS (Fig. 14b).

Teichoic acid biosynthesis. A carrier lipid also appears to participate in synthesis of a polymer of the teichoic acid type in *Staphylococcus lactis* (5, 14). The final product is a repeating polymer of *N*-acetylglucosamine-α,1-phosphate, $(\text{GlcNAc} \xrightarrow{\alpha} \text{P-GlcNAc} \xrightarrow{\alpha} \text{P-})_n$, and the details of the reaction will be of great interest because of the formation of a phosphodiester bond in the product.

Membrane fragments of the organism catalyze the incorporation of ^{14}C-GlcNAc from UDP-GlcNAc into a butanol-extractible product which appears to be GlcNAc-P-P-lipid, although the product has not yet been completely characterized. The nucleotide product of this reaction is UMP and the reaction is easily reversible, thus corresponding to the initial reactions in the synthesis of peptidoglycan and O-antigen.

Preliminary evidence that GlcNAc-P-P-lipid is an intermediate in synthesis of the poly-(GlcNAc-P) was obtained by pulse-labeling experiments. These indicated that an increase in ^{14}C-GlcNAc in the polymer occurred during a period when radioactivity in ^{14}C-GlcNAc-P-P-lipid was decreasing.

Biosynthesis of capsular polysaccharide in Aerobacter aerogenes. A capsular polysaccharide of *A. aerogenes* contains the oligosaccharide repeating unit Gal-(GlcUA)-Man-Gal in which the glucuronic acid residue occurs as a single branch on the mannosyl residue of every repeating unit. Evidence for a lipid-linked intermediate in the biosynthetic pathway was obtained by incubating a cell envelope fraction with UDP-^{14}C galactose and demonstrating that ^{14}C-galactose appeared in a chloroform-methanol-soluble product whose properties were similar to Gal-P-P-GCL (17). This reaction was reversed by UMP, as expected from other GCL-linked pathways. Mannose could then be incorporated from GDP-mannose into the lipid-soluble radioactive product. Addition of GDP-mannose and UDP-glucuronic acid caused the radioactivity to appear in the polysaccharide, suggesting that the lipid-linked oligosaccharide was a true intermediate in synthesis of the polymeric capsular polysaccharide.

Evidence for the following pathway was obtained by the sequential addition of mannose, glucuronic acid, and galactose to the original Gal-P-P-GCL formed in the initial reaction.

$$\text{UDP-galactose} + \text{P-GCL} \rightleftarrows \text{Gal-PP-GCL} + \text{UMP} \quad \text{XII}$$

$$\text{GDP-mannose} + \text{Gal-P-P-GCL} \rightarrow \text{Man-Gal-PP-GCL} + (\text{GDP}) \quad \text{XIII}$$

$$\text{UDP-glucuronate} + \text{Man-Gal-P-P-GCL} \rightarrow \text{GlcUA-Man-Gal-PP-GCL} + (\text{UDP}) \quad \text{XIV}$$

$$\text{UDP-galactose} + \text{GlcUA-Man-Gal-P-P-GCL} \rightarrow \text{Gal(GlcUA)Man-Gal-PP-GCL} + (\text{UDP}) \quad \text{XV}$$

The polymerization step is initiated while the sugars are still linked to the carrier lipid and octasaccharide-P-P-GCL has been isolated. However, it is not yet clear whether further polymerization beyond this stage also occurs while the chain is still lipid-linked. The carrier lipid was identified as a C_{55} polyisoprenoid alcohol and, therefore, the system seems analogous to the O-antigen synthesizing system (17).

Metabolism of Glycosyl Carrier Lipids

Biosynthesis of glycosyl carrier lipid. All glycosyl carrier lipids that have been identified are polyisoprenoid alcohol phosphates containing 11 unsaturated isoprene units with a terminal ester-linked phosphate residue. In *Salmonella newington*, biosynthesis of GCL appears to proceed by the condensation of farnesyl pyrophosphate and Δ^3-isopentenyl pyrophosphate with elimination of inorganic pyrophosphate (13; Fig. 15). The successive addition of isoprene residues by this mechanism leads to formation of the final polyisoprene chain, terminated in a pyrophosphate residue. The final reaction is presumed to be hydrolysis of the pyrophosphate to form P-GCL + Pi.

Christensen et al. (13) demonstrated that extracts of *S. newington* will catalyze the synthesis of functional carrier lipid from ^{3}H-farnesyl pyrophosphate and ^{14}C-Δ^3-isopentenyl pyrophosphate (13). Identification of the product as P-GCL was obtained by incubating the reaction mixture with TDP-rhamnose and UDP-galactose in the presence of a crude membrane fraction. This led to formation of Rha-Gal-lipid, which was chromatographically indistinguishable from authentic Rha-Gal-P-P-GCL. The disaccharide-lipid contained both ^{3}H and ^{14}C, indicating that the isopentenyl and farnesyl groups were both incorporated into the product (Fig. 15). It should be noted that the presumed product of the condensation reactions is PP-GCL and that hydrolysis to P-GCL must occur before

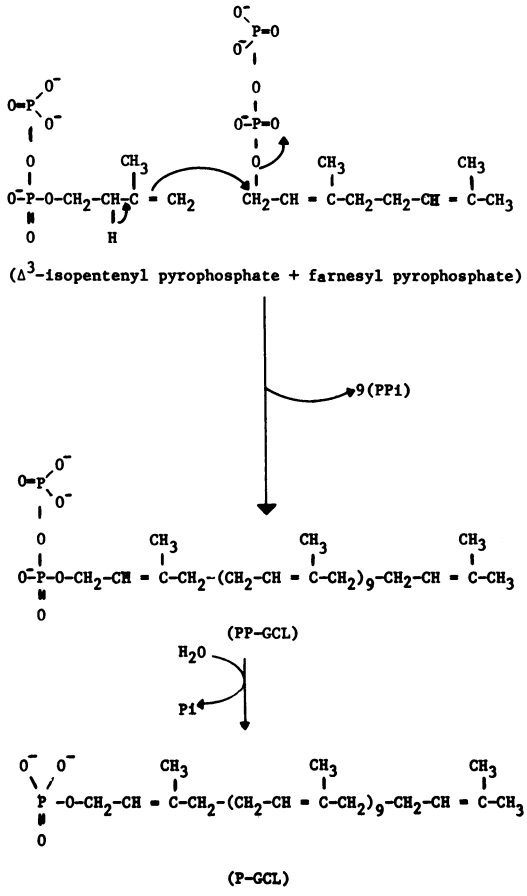


FIG. 15. *Proposed pathway of biosynthesis of P-GCL in Salmonella newington (13).*

the lipid can participate in the O-antigen synthesis cycle.

Although all glycosyl carrier lipids thus far studied have chain lengths of 11 isoprene units, the in vitro preparation leads to formation of products with a range of short chain lengths in addition to the longer chain length product that appears to be GCL. The short chain length compounds were shown to be produced by enzyme activities present in the soluble fraction of the broken cell preparation. In contrast, the particulate cell envelope fraction catalyzed formation of a product that was similar to authentic GCL both on thin-layer chromatography and when assayed for ability to participate in formation of the disaccharide-lipid (Rha-Gal-lipid). Thus, synthesis of GCL may take place directly in the membrane. The fate of the shorter chain compounds produced by the soluble fraction is not known.

Dephosphorylation and rephosphorylation of PP-GCL (Fig. 16). The peptidoglycan and O-antigen-synthesizing reactions lead to formation of lipid-pyrophosphate (PP-GCL) which must then be converted to lipid-monophosphate (P-GCL) to participate in another turn of the biosynthetic cycle (reaction 6 in Fig. 11, 12, and 16).

This represents one potential site for the regulation of the synthesis of peptidoglycan or O-antigen. Preliminary evidence indicates that the membrane fraction of *S. aureus* contains an enzyme which catalyzes this dephosphorylation reaction. The dephosphorylation reaction is inhibited by bacitracin, thereby causing accumulation of PP-GCL and preventing reutilization of P-GCL in the next turn of the cycle (72). This results in inhibition of peptidoglycan synthesis, although it is possible that de novo formation of P-GCL could permit a low level of synthesis to continue. However, as noted above, de novo synthesis may also require the dephosphorylation reaction since the product of the biosynthetic pathway appears to be PP-GCL rather than P-GCL, and de novo synthesis may therefore be expected to be affected by bacitracin. As expected (*see* Fig. 12), synthesis of O-antigen is also blocked by bacitracin (45) since the conversion of PP-GCL to P-GCL is required for the initial step in the cycle. Thus, the synthesis of both peptidoglycan and O-antigen can be regulated by controlling the rate of regeneration of P-GCL.

Another possible mechanism of control involving P-GCL has been demonstrated in *Staphylococcus aureus*. An enzyme activity in the membrane fraction of this organism catalyzes the phosphorylation of polyisoprenoid alcohols with formation of polyisoprenoid alcohol monophosphates (19).

$$\text{GCL} + \text{ATP} \rightarrow \text{P-GCL} \ (? + \text{ADP}) \qquad \text{XVI}$$

This was an unexpected finding since P-GCL and not GCL is the product of the biosynthetic cycle. In addition, the GCL biosynthetic pathway leads to formation of PP-GCL rather than GCL. Thus, the presence of the phosphorylating enzyme implies that PP-GCL or P-GCL can be dephosphorylated to GCL within the cell. Since GCL cannot enter the biosynthetic cycle, this would effectively reduce the available carrier lipid and thereby decrease the rate of the biosynthetic reactions. The kinase reaction (equation XVI) results in reformation of the active compound P-GCL and should, therefore, lead to an increase in the rate of peptidoglycan synthesis. This explains the previously unexplained finding that the rate of peptidoglycan synthesis in the *S. aureus* system was stimulated by adenosine triphosphate (ATP), although ATP did not

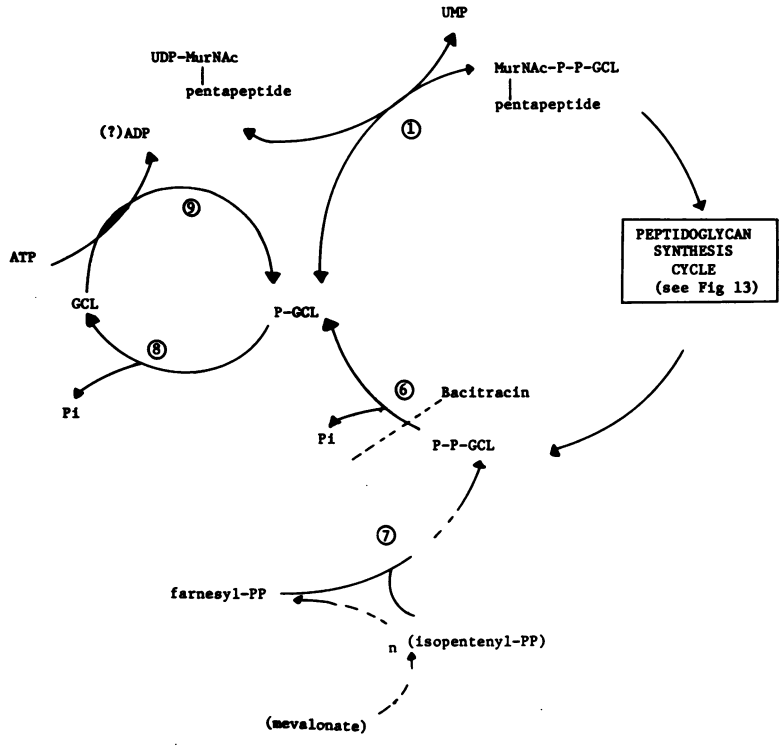


FIG. 16. *Metabolism of P-GCL in Staphylococcus aureus.*

appear to be required in any of the individual reactions shown in Fig. 11.

The product of the kinase reaction (reaction 9 in Fig. 16) appears capable of functioning as carrier lipid in the peptidoglycan system when C_{55} ficaprenol [HO—(CH_2—CH=$C(CH_3)$—CH_2)$_{11}$H] is used as the phosphate acceptor. A ^{32}P-ficaprenol product was prepared in the kinase reaction, using AT^{32}P as the phosphate donor. When the product was incubated with UDP-MurNAc-pentapeptide or with UDP-MurNAc-pentapeptide plus UDP-GlcNAc, in the presence of a butanol-treated enzyme preparation, new radioactive compounds were formed which were chromatographically indistinguishable from authentic MurNAc-pentapeptide-P-P-GCL and GlcNAc-MurNAc-pentapeptide-P-P-GCL (19).

The enzyme (isoprenoid alcohol phosphokinase) requires phospholipid as a cofactor and, therefore, falls in the general category of lipid-activated membrane enzymes. Phosphatidylglycerol and cardiolipin of bacterial origin are quite specific in this regard, and little activity was seen with other phospholipids (20). It appears that shorter chain length polyisoprenoid alcohols ($<C_{55}$) can also serve as substrates for the kinase, but the details of the substrate specificity remain to be fully defined. It is not known whether isoprenoid alcohol phosphates with chain lengths other than C_{55} can function as carrier lipids in the peptidoglycan and O-antigen systems.

The kinase also has the unusual property of being solubilized when the membrane is treated with acidic *n*-butanol at room temperature. The butanol extract contains all three components of the system, i.e., enzyme, phospholipid, and C_{55}-isoprenoid acceptor. Further fractionation on diethylaminoethyl-cellulose in organic solvents resulted in separation of the enzyme from phospholipid and permitted study of the specific lipid requirements of the system (20).

The role of the polyisoprenoid alcohol kinase in the normal physiology of the cell has not yet been defined. It should be noted that the kinase system may be unique to *S. aureus* since ATP stimulation of peptidoglycan synthesis has not been observed in peptidoglycan-synthesizing preparations from other organisms.

Role of Carrier Lipid-Linked Pathways

What are the possible advantages to the cell in having the GCL-linked synthetic pathways?

It was originally suggested by Anderson et al. (1) that the role of carrier lipid is to transport the oligosaccharide precursors of peptidoglycan (i.e., GlcNAc-MurNAc-pentapeptide) across the membrane. The nonpolar nature of GCL would facilitate this transfer. In this scheme, all of the synthetic reactions which require water-soluble substrates could occur on the inner surface of the cytoplasmic membrane, leaving only the transpeptidation reaction, which requires no additional energy, to take place outside of the membrane. A similar role for GCL could be postulated in the O-antigen system. In addition, two additional possibilities can be suggested. (i) The LPS acceptor of O-antigen transfer is a membrane component and it would be advantageous for the O-antigen-synthesizing system to be located in close proximity to the final acceptor to facilitate the final transfer reaction. Carrier lipid, by being located in the membrane adjacent to the LPS acceptor, could serve this role without postulating any transmembrane transport function. In addition, the model proposed for synthesis of the core LPS (*see* Some Speculations) may also apply to O-antigen synthesis. If so, GCL, by virtue of its nonpolar nature, may serve a true carrier function by permitting the growing O-antigen chain to move from enzyme to enzyme within the membrane structure (i.e., from UDP-Gal transferase to TDP-Rha transferase to GDP-Man transferase to O-antigen polymerase to ligase, as indicated in Fig. 12). Similar reasoning would apply to other cell envelope polymers whose synthesis involves GCL. (ii) If O-antigen synthesis occurs in the cytoplasmic membrane, a transport mechanism must exist to transfer either $(\text{oligosaccharide})_n$-P-P-GCL or completed LPS to the outer membrane, as discussed above. The carrier lipid could be involved in this final translocation process.

Clearly these three possible functions are not mutually exclusive, and GCL may play more than one role in the normal process of cell growth and morphogenesis.

As shown in Fig. 11, 12, and 16, there are various potential sites of control of the synthesis of polymers whose biosynthetic pathways utilize the carrier lipid mechanism. On the other hand, nothing is now known about the physiological regulation of the reactions or of the possible functional interrelationships between these synthetic pathways and the degradative reactions which are presumed to operate in cell growth and cell division. The genetic loci controlling synthesis of many of the enzymes responsible for these reactions have been characterized, but the genetic control mechanisms which operate in these systems are still largely unexplored. Thus, various important questions are now open for investigation, and the answers should be of great interest in the areas of cell growth, morphogenesis, and division.

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